Proteomic analysis of IgM antigens from mammary tissue under pre- and post-cancer conditions using the MMTV-PyVT mouse model

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Abstract

We analyzed the recognition of tumor antigens by IgM in transgenic MMTV-PyVT mice. PyVT female mice are a model of breast cancer that simulates its counterpart in humans. The PyVT model allows studying antigen recognition in two conditions: before and during tumor expression. We attempted to identify by sequence, the antigens recognized by IgM that are expressed or disappear in the membrane of breast transgenic tissue during the transition "No tumor-Tumor". 2D immunoblots were obtained of isolated membranes from the breast tissue in the fifth, sixth, and seventh week (transition point). Proteins recognized by IgM were sequenced in duplicate by MALDI-TOF. In the transition, we observed the disappearance of antigens in transgenic mice with respect to non-transgenic ones. We believe that in the diagnosis of cancer in its early stages, the expression of early antigens is as important as their early delocalization, with the latter having the advantage that, under normal conditions, we can know which proteins should be present at a given time. Therefore, we could consider that also the absence of antigens could be considered as a biomarker of cancer in progress

Introduction

Tumors express aberrant, mutated, or modified proteins that are associated with malignant growth. These proteins are known as tumor-associated antigens and the carbohydrates in these proteins are tumor-associated carbohydrate antigens. These antigens can stimulate the humoral and cellular immune responses [1,2]. Carbohydrate epitopes are stably expressed in many tumors at various precursor stages. Unlike polypeptide chains, glycoepitopes share structural homologies

39 beyond the boundaries of protein families; therefore, they can cross-react and be prime targets
40 for natural IgM antibodies [3].
41 Two types of IgM can be found in the blood of normal mice: Natural IgM, secreted by CD51 B-1
42 cells in the absence of antigenic stimulation and which concentrates most of the circulating IgM.
43 On the other hand, antigen-induced IgM is produced primarily by B cells (B-2) only after
44 antigenic stimulation. Both natural and induced IgM are polymeric and efficiently activate the
45 classical complement cascade. Natural antibodies exhibit a broad spectrum of antibacterial
46 activity and serve as the first line of defense against microbial and viral infections [4,5]. Besides,
47 IgM is associated with the recognition and elimination of precancerous and cancerous cells [6-8].
48 Previously, we demonstrated the advantage of IgM with respect to IgG in the recognition of
49 tumor antigens [9,10]. In that paper, variability in the IgM response was manifested as a pattern
50 of spots could represent a signature of the immune response. Different numbers of spots was
51 found in the IgG and IgM responses. On average, the IgM had more and stronger response than
52 IgG but also the latter decrease with the time. In this context our question was: In an organism
53 that we know will have breast cancer, will its pattern of antigenic recognition by
54 immunoglobulin M vary at the time of transition?
55 For several years, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), followed by
56 mass spectrometry for identifying proteins, has been the main technique for the discovery of
57 biomarkers in conventional proteomic analysis [11,12]. This technique is especially suitable for
58 making direct comparisons of the differential expression of proteins between normal and tumor
59 tissues [13-19]. Recently, NMR metabolomics to a small blood sample offers a complementary
60 approach to the current pathway for investigating patients with a clinical suspected possible
61 malignancy. It is sensitive, specific, and of low cost, requiring nothing more than a blood sample
62 in the clinic and an inexpensive NMR analysis, and can identify patients with solid tumors when
63 referred with nonspecific symptoms (20)
64 Mouse Mammary Tumor Virus promoter-Polyoma Virus Middle T Antigen (MMTV-PyVT)
65 mice is a murine model that has been genetically modified to develop breast cancer with
66 characteristics similar to human breast cancer. We believe that, with this model, we can achieve
67 our goal of comparing the pattern of antigen recognition by the IgM in two conditions: before
68 and during the expression of the tumor. In the present study, we report the pattern of antigenic
69 recognition by IgM in serum of transgenic or non-transgenic mice with the membrane fraction of
70 breast tissue at the sixth week, which is the starting period of the transformation process.
71 Therefore, we sequenced the antigens recognized at that time shortly after the Middle T Antigen
72 (MT) antigen gene was expressed.
73

74 **Materials & Methods**
75 Ethics statement
76 Animal care and experimental practice were conducted at the Unidad de Modelos Biológicos
77 (UMB) in the Instituto de Investigaciones Biomédicas (IIB), Universidad Nacional Autónoma de
78 México. All experimental procedures in the animals were approved by the Institutional Care and

79 Animal Use Committee (CICUAL), permit number ID 264, adhering to Mexican regulation
80 (NOM-062-ZOO-1999), and in accordance with the recommendations from the National
81 Institute of Health (NIH) of the United States of America (Guide for the Care and Use of
82 Laboratory Animals), available online at: https://www.biomedicas.unam.mx/servicios/unidad-de-
83 modelos-biologicos. Euthanasia of experimental animals and controls was performed humanely
84 by cervical dislocation after anesthesia with 5% sevofluorane (Abbot, México).

85 Animals
86 Transgenic male FVB/N-Tg(MMTV-PyVT) 634Mul/J mice and FVB/N female mice were
87 purchased from Jackson Laboratories (USA). The animals were housed at UMB at a controlled
88 temperature (22°C) and 12-hr light-dark cycles, fed with water and Purina LabDiet 5015 (Purina,
89 St. Louis MO) chow ad libitum, bred in the animal facilities at the UMB, and placed in standard
90 cages with 5 mice per cage. The experimental design considers one independent variable: the
91 antigen recognition pattern during mammary tumor induction (non-transgenic control vs
92 transgenic group). Therefore statistical analysis is not applicable. Four transgenic female mice
93 and Four non transgenic female mice were used. The animals in the different experimental
94 groups were treated and assessed at the same time. No randomisation was used to allocate
95 experimental units to no transgenic and transgenic groups. The study did not have humane
96 endpoints,

97 DNA Extraction and PCR
98 A 0.5 cm segment of tail tissue was cut from the 3-week-old mice. The tissue was processed
99 according to the instructions of the DNA Wizard® Genomic DNA Purification Kit
100 (PROMEGA). Transgenic mice were recognized by PCR using the following primers for PyVT
101 oncogene: 5′GGGAAGCAAGTACTTCACAAGG3′ (forward) and
102 5′GGAAAGTCACTAGGAGCAGGG3′ (reverse). As load control, β actin primers
103 5′GGAGGTCATCACTATTGGCAACGAG3′ (forward) and
104 5′TGTTTACGGATGTCAACGTCACACT3′ (reverse) were employed.

105 Serum
106 Sera from individual mice were obtained at the fifth, sixth, and seventh week . We incubated the
107 blood at 4°C for 30 minutes and then centrifuged it to obtain the serum. The sera were stored at -
108 20°C until used.

109 Isolation of the membrane fraction of the Breast Tissue
110 Breast tissue at fifth, sixth, and seventh week was homogenized in PBS pH 7.4 in the presence of
111 a protease inhibitor. The homogenate was centrifuged at 14 000 G for 15 min. The supernatant
112 was transferred to a polycarbonate tube and centrifuged in a 90Ti rotor (Beckman) at 100,000 G
113 for 1 hour at 4oC. The pellet was resuspended in lysis buffer for 2D Electrophoresis: 6 M urea,
114 50 mMDTT, 2% CHAPS in the presence of a protease inhibitors cocktail (Thermo Scientific).

115 2D Electrophoresis
116 For this part, the methodology described in Díaz-Zaragoza et al. [9] was followed.

117 Mass spectrometry and protein identification

118   The samples were prepared for mass spectrometry analysis with a slight modification of a
119   previously described procedure [21]. The data processing software used was Protein Lynxs
120   Global Server 2.5.1 (TM Waters). The base of Mus musculus, extracted from UNIPROT, was
121   used for the identification of proteins.
122

123   **Results**

124   Figure 1 shows a representative amplification of MT oncogene in the newborn positive female
125   mice selected for subsequent experiments. During the first six weeks, the mice were active and
126   normal in appearance. As the 9th week approached, mobility decreased.
127   To identify antigens recognized by IgM shortly after the MT gene was expressed (sixth week),
128   the membrane proteins of both normal and transgenic mammary tissue were separated in the
129   fifth, sixth, and seventh weeks (there were only differences in the recognition pattern in the sixth
130   week; therefore, the analysis was focused on this time). At the sixth week, there was variability
131   in the antigen recognition pattern; each female presents its own antigenic pattern. However, in all
132   cases it was observed that some antigens were recognized in the non-transgenic female but not in
133   the transgenic female. For example, in figure 2, four conspicuous antigens can be observed,
134   which were recognized by the IgM of the normal female and were not recognized by the
135   transgenic female. These four antigens caught our attention and were sequenced in duplicate for
136   identification.
137

138   Table 1 shows the identified proteins that coincided in the sequencing of two independent
139   samples. Spots A, B, C, and D were sequenced in duplicate and the identification results are
140   presented. The sequencing of spot A resulted in a protein that is encoded by the 2210010C04Rik
141   gene and that corresponds to a Peptidase S1 domain-containing protein. The sequencing of spot
142   B yielded two consensus proteins in the two independent analyses: a Peptidase S1 domain-
143   containing protein and Keratin 78. Spot C also yielded two consensus proteins: Keratin type II
144   cytoskeletal 73, and Keratin type I cytoskeletal 10. Spot D gave one consensus protein: Keratin
145   type II cytoskeletal 6B. At the sixth week, normal breast tissue expresses keratins related to the
146   cytoskeleton and proteases in its membrane.
147

148   **Discussion**

149   The transgenic mice FVB/N-Tg (MMTV-PyVT) 634Mul/J (transgenic mice PyVT) express the
150   oncogene of Middle T Antigen (MT), which induces the transformation of cells from mammary
151   tissue to a tumorigenic state; thus, the mice have a predisposition to developing breast cancer
152   [22]. Zagozdzon et al. showed that, in transgenic PyVT mice, the expression of the MT gene
153   begins at the age of 5 weeks, thus initiating the tumor process [23]. The membranal fraction of
154   the mammary tissue at the fifth week did not show differences in antigenic identification by IgM
155   (data not shown), but at the sixth week, it did. The results on the recognition of antigens
156   distinguished by IgM one week after the MT gene was expressed can be explained because the
157   MT antigen, when expressed, inserts into the membrane due to a contiguous 22 amino acid

158 hydrophobic segment near the carboxyl terminal. The MT antigen has been shown to interact
159 with members of the Src family of tyrosine kinases, phosphoinositol-3-kinase (PI-3-kinase), and
160 phosphatase 2A, altering their activity and localization. These proteins, in turn, play a role in
161 cytoskeletal regulation, since the expression of the MT antigen generates profound changes in
162 actin stress fibers, focal adhesions, and the regulation of microtubule arrangement. Our results
163 confirm that the interaction of MT with membranes may involve components of the
164 cytoskeleton. The non-detection of membrane proteins by the IgM of transgenic females is
165 therefore explained by the alterations in cell morphology that accompany the transformation (24-
166 27).
167 In the genesis of normal breast tissue, there are structural, compositional, and functional changes
168 during an individual's life. The mammary epithelium undergoes dramatic growth, differentiation,
169 and function responses to hormonal stimuli during the four stages of the mammary development
170 cycle represented in virgin, pregnant, lactating, and involutional animals. Primary glandular
171 branching morphogenesis occurs at approximately five weeks in mice, integrating epithelial cell
172 proliferation, differentiation, and apoptosis. Sexual maturity is reached at approximately five
173 weeks in mice, although the ductal network continues to grow beyond this point until it reaches
174 its full dimension at around eight weeks (28). The antigens identified by the IgM of the normal
175 female correspond to this stage of tissue remodeling (5-8 weeks); therefore, the sequenced
176 proteins are part of this enzymatic-structural complex that breast tissue undergoes at the sixth
177 week of age. Both the proteases and keratins found have been reported in the remodeling of the
178 extracellular matrix during the different stages of mammary development and, therefore, in the
179 modulation of the function of mammary cells (29-33).
180 It cannot be ruled out that the disappearance of the antigens is due to another reason related to
181 the health of the mouse and not necessarily to cancer in progress, however we consider that
182 although it could be possible we consider it to be unlikely
183

Conclusions

185 In the search for biomarkers that are related to the initial stages of cancer, it is common to try to
186 identify proteins that are modified by the carcinogenesis process or that are expressed de novo.
187 However, the absence of a protein can also be a biomarker. In other words, the cell, in its
188 transformation process, presents an imbalance of its normal protein synthesis machinery and an
189 absence of a protein could be indicative of a neoplasm on the way. In the case of breast tissue,
190 the stages of development are known in detail (both in mice and in humans), so in the
191 transformation process, apparently due to a reorganization of the membrane proteins (see
192 references 25,26,28,33) the proteins that normally should be in the membrane are no longer there
193 and that this could be a way of recognize, identify or predict an impending oncogenesis process.
194 It is necessary to say that we are not proposing the proteins identified here as biomarkers of the
195 beginning of a transformation process. About keratins and breast cancer has already been
196 written. More studies need to be done to make claims to that effect.
197

198 **Acknowledgements**

199

200 **References**

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287

Figure 1

Identification by PCR

Fig. 1. Identification by PCR of newborn female PyVT mice carrying the MT oncogene. The gel shows representative results for positive female mice (A and C) and negative female mice (B) for MT oncogene amplification.

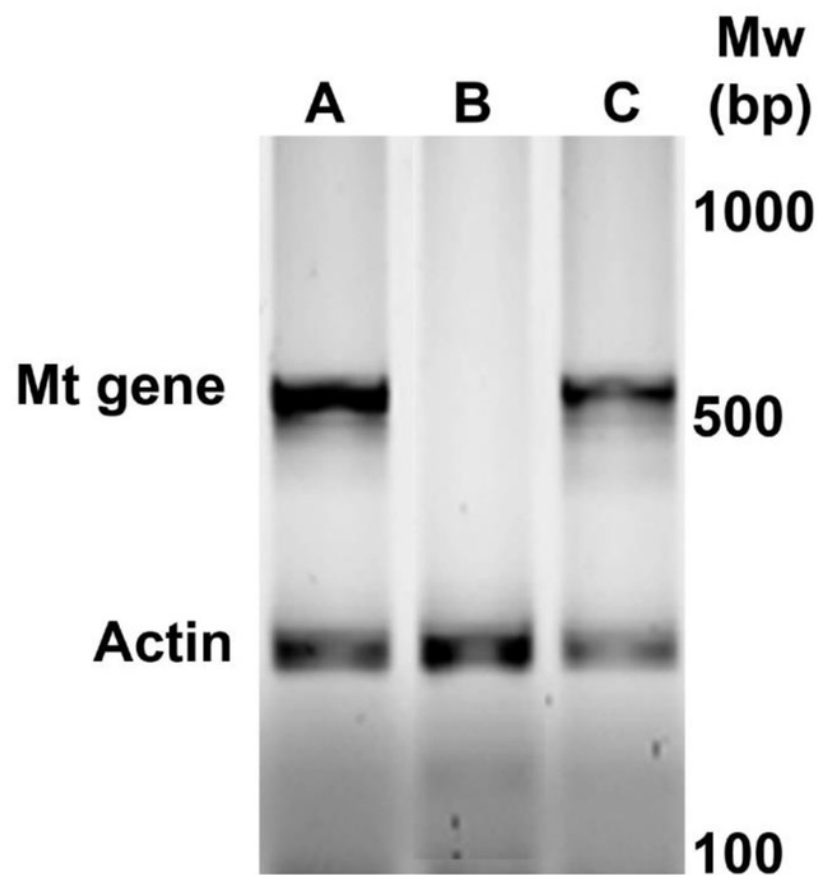



Figure 2

Loading control

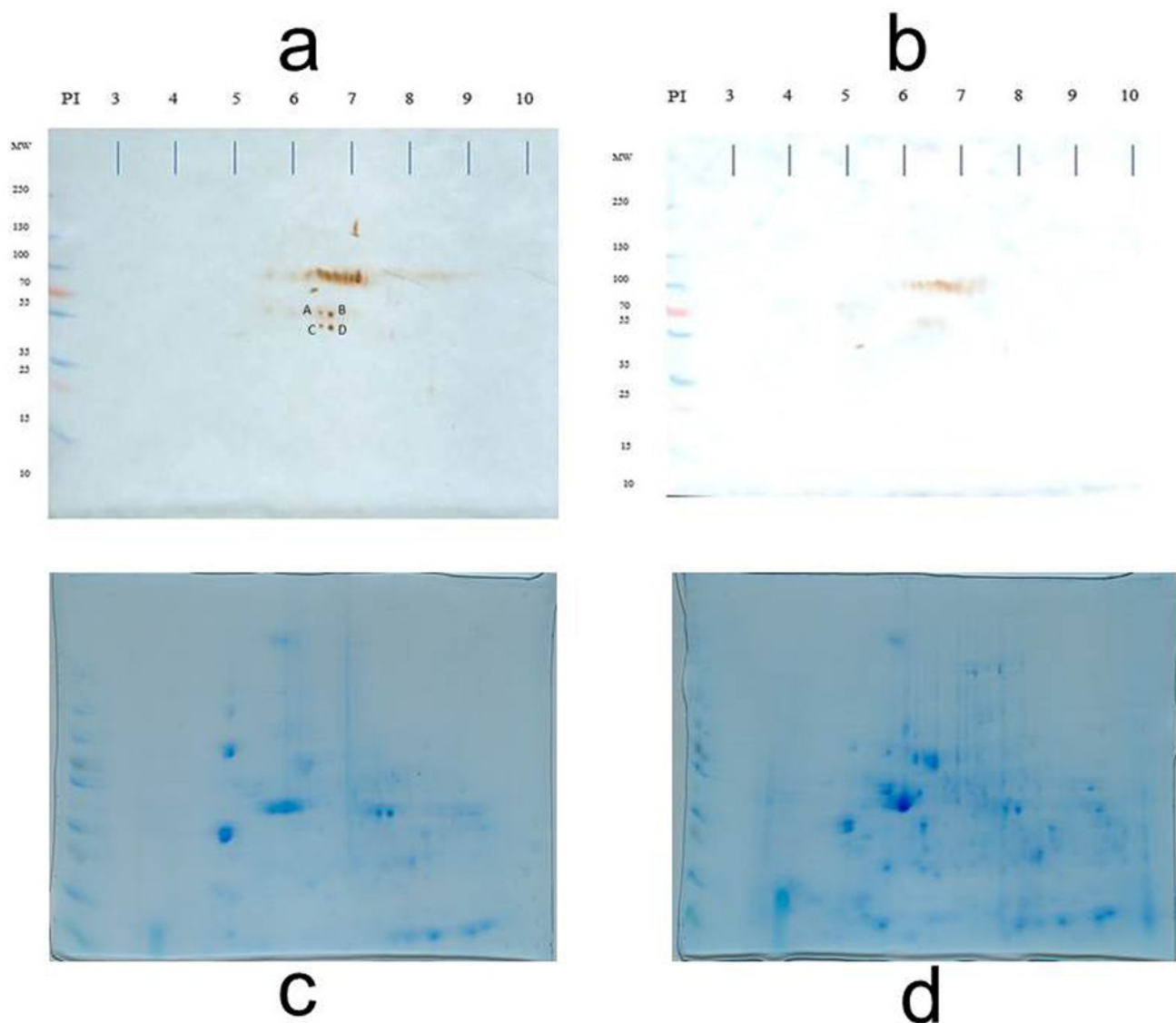
Figure 2. Representative 2D immunoblot of antigenic recognition by serum IgM of normal female mice (a) and Transgenic mice (b) on the membrane fraction of mammary tissue at the sixth week of age. Blots c and d represent the loading control of immunoblots a and b, respectively. Blots c and d are stained according to as indicated in materials and methods (ref. 9)

corrected table

Table 1. Identification of spots A, B, C, D of figure 2. The sequences are the consensus proteins of two independent analyzes

SPOT	ACCESSION	DESCRIPTION
A	Q9D7Y7_MOUSE	Peptidase S1 domain-containing protein
B	Q9CPN9_MOUSE	Trypsinogen 7
B	E9Q0F0_MOUSE	Keratin 78
C	K2C73_MOUSE	Keratin, type II cytoskeletal 73
C	K1C10_MOUSE	Keratin, type I cytoskeletal 10
D	K2C6B_MOUSE	Keratin, type II cytoskeletal 6B

7 Table 1. Identification of spots A, B, C, D of figure 3. The sequences are the consensus
8 proteins of two independent analyzes